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ORIGINAL REPORT

Postinduction Minimal Residual Disease Monitoring by Polymerase Chain Reaction in Children With Acute Lymphoblastic Leukemia

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ABSTRACT

Purpose
Acute lymphoblastic leukemia (ALL) is the most common pediatric cancer. Monitoring minimal residual disease (MRD) by using real-time quantitative polymerase chain reaction (RQ-PCR) provides information for patient stratification and individual risk-directed treatment. Cooperative studies have documented that measurement of blast clearance from the bone marrow during and after induction therapy identifies patient populations with different risk of relapse. We explored the possible contribution of measurements of MRD during the course of treatment.

Patients and Methods
We used RQ-PCR to detect MRD in 110 unselected patients treated in Italy in the International Collaborative Treatment Protocol for Children and Adolescents With Acute Lymphoblastic Leukemia (AIEOP-BFM ALL 2000). The trial took place in AIEOP centers during postinduction chemotherapy. Results were categorized as negative, low positive (below the quantitative range [$< 5 \times 10^{-4}$]), or high positive ($\geq 5 \times 10^{-4}$). Patients with at least one low-positive or high-positive result were assigned to the corresponding subgroup.

Results
Patients who tested high positive, low positive, or negative had significantly different cumulative incidences of leukemia relapse: 83.3%, 34.8%, and 8.6%, respectively ($P < .001$). Two thirds of positive cases were identified within 4 months after induction-consolidation therapy, suggesting that this time frame may be most suitable for cost-effective MRD monitoring, particularly in patients who did not clear their disease at the end of consolidation.

Conclusion
These findings provide further insights into the dynamic of MRD and the ongoing effort to define molecular relapse in childhood ALL.

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INTRODUCTION

Acute lymphoblastic leukemia (ALL) is the most common type of cancer in children and adolescents. With current risk-directed treatment approaches, cure rates exceed 80%.[1] Leukemia relapse remains the most common cause of treatment failure. Although a proportion of patients may be rescued after relapse, second-line therapy may be ineffective in most of the cases, despite intensive approaches, including allogeneic hematopoietic stem-cell transplantation (HSCT).[2,3] Minimal residual disease (MRD) monitoring is currently considered the most reliable strategy to evaluate early treatment response and to refine stratification accordingly.[4-9] Both highly sensitive molecular polymerase chain reaction (PCR) and multiparameter flow cytometry approaches may be used to this purpose in the context of first-line, relapse, or allogeneic HSCT trials.[10-17] The use of immune gene rearrangements by real-time quantitative PCR (RQ-PCR) allows stratification of approximately 90% of the patients with a single sensitive marker.[17]

These techniques allow identification of insufficient blast cell clearance during initial treatment with disease persistence and also allow identification of the re-emergence of leukemic cells, thus challenging the criteria for the definition of remission[18,19] or medullary relapse, which, at this point, are based on standard morphologic evidence of 25% leukemic blasts in the bone marrow

(BM) in a patient who previously achieved morphologic complete remission (CR).

Despite adequate technologies that are now widely available, only a few studies have addressed the issue of predicting ALL relapse by prospective, postinduction MRD monitoring. Conversion to MRD positivity during early postconsolidation treatment in adult patients with standard-risk ALL (SR-ALL) was strongly predictive of subsequent morphologic relapse.[20] Although no interventional strategies have been applied so far in ALL, the experience of the Gruppo Italiano Malattie Ematologiche Maligne dell'Adulto in patients with acute promyelocytic leukemia for which the administration of salvage therapy at the time of molecular relapse improved the chance of rescuing patients[21] might suggest a potential benefit.

In this study, we addressed this issue by monitoring MRD throughout the treatment program in children with ALL treated with a Berlin-Frankfurt-Münster (BFM) –type intensive chemotherapy.

PATIENTS AND METHODS

Patients in this study were newly diagnosed with childhood ALL between September 2000 and July 2006 and were enrolled onto the International Collaborative Treatment Protocol for Children and Adolescents With Acute Lymphoblastic Leukemia (AIEOP-BFM ALL 2000).[6,10] AIEOP-BFM ALL 2000 trial patients from two AIEOP centers were eligible for our study.

Patient Stratification

The definitions for MRD risk are as follows: SR-MRD: MRD was negative at day 33 (time point 1 [TP1]) and day 78 (TP2) by using at least two molecular targets with a sensitivity $\geq 1 \times 10^{-4}$; intermediate-risk MRD (IR-MRD): MRD was positive at less than 5×10^{-4} at TP2; high-risk MRD (HR-MRD): MRD was $\geq 5 \times 10^{-4}$ at TP2. Patients with either prednisone-poor response ($\geq$ 1,000 circulating blasts per microliter on day 8) or patients who did not achieve CR after induction phase IA or who had translocation t(4;11) were all allocated to the HR group, independently of MRD results.

Treatment Protocol

Treatment was administered as reported elsewhere.[6,10] Briefly, all patients were given a 7-day steroid prephase, induction protocols IA and IB, followed by consolidation with high-dose methotrexate for non–high-risk patients or block therapy for high-risk patients, then by reinduction and by maintenance until 24 months from diagnosis. Treatment details, including randomizations, are provided in the Appendix (online only).

BM Sampling

All patients underwent surveillance BM aspirate at the beginning of protocol IB, protocol M or first block, protocol II or III, and continuation therapy.

Patients in our study underwent additional surveillance aspirates approximately every 2 months during continuation therapy until month +24 from diagnosis and 2 months after treatment discontinuation. Material from each TP was used for MRD determination. Collected samples were batched and analyzed later on; treating physicians were blinded to the results.

Institutional review board approval for this trial was obtained locally by each participating institution. Informed consent for MRD evaluation on any BM aspirate was obtained from the parents or legal guardians.

Study Population

In all, 276 Philadelphia chromosome–negative patients were enrolled onto the AIEOP-BFM ALL 2000 trial in two centers that adopted the additional MRD surveillance. Fifty-five were not eligible for this study because of induction failure (n = 6) or lack of a marker by RQ-PCR with a sensitivity $\geq 10^{-4}$ (n = 49). This proportion of ineligible patients is comparable to that in the overall study.[6,10] Of 221 eligible patients, 110 accepted surveillance BM sampling.

Statistical Analysis

χ^2 or Fisher's exact tests were used to evaluate the association between the frequency of main characteristics and that of MRD positivity during monitoring. Disease-free survival (DFS) and cumulative incidence of relapse (CIR) were the main end points for outcome analysis. DFS was defined as the time from diagnosis to first treatment failure, which was defined as relapse, death in remission, or development of second malignant neoplasm. Observation of patients was censored at the time of last contact, when no events were observed. The Kaplan-Meier method was used to estimate probabilities of DFS with SEs calculated according to Greenwood's formula and CIR (because no competing events were observed). The Cox regression analysis was performed on the cause-specific hazard of relapse to evaluate the MRD profile after adjusting for risk group. Analyses were carried out by using SAS 9.2 (SAS Institute, Cary, NC).

DNA Isolation

Mononuclear cells were isolated by Ficoll-Paque gradient centrifugation, and DNA was extracted and purified by using the GentraPuregene DNA Purification Kit (Gentra Systems, Monza, Italy) according to the manufacturer's instructions.

Identification of PCR Targets and Design of Allele-Specific Oligonucleotides

Genomic DNA samples obtained at diagnosis and at relapse were screened for clonal immunoglobulin H/K chain, T-cell receptor gene rearrangements, and SIL-TAL by using published primer sets.[17,22,23] Clonal immune gene rearrangements were identified by heteroduplex analysis and sequenced by using the BigDye Terminator Cycle Sequencing Kit with the ABIPrism 310 Genetic Analyzer (Applied Biosystems, Foster City, CA). After sequencing, allele-specific oligonucleotides were designed for each PCR target based on the sequence data of the junctional region by using Primer Express software v3.0 (Applied Biosystems).

Evaluation and Interpretation of MRD RQ-PCR Results

The designed allele-specific oligonucleotides were then tested in combination with germ-line primers and TaqMan probes by RQ-PCR using the 7900HT Sequence Detection System (Applied Biosystems). PCR analysis was then performed and results were interpreted according to the guidelines developed by the European Study Group on MRD Detection in ALL (EuroMRD) to reduce the risk of false-negative and false-positive results.[24] Briefly, three replicates were performed for MRD analysis for each TP; 500 ng of DNA per 25 μL was used for each reaction; six replicates of the polyclonal control were used to define the background amplification. Although two informative markers were mandatory for MRD stratification by study design in the AIEOP-BFM ALL 2000 trial, our study included patients with a single informative marker.

Surveillance MRD results were categorized as MRD negative in the absence of specific amplification or amplification within three threshold cycles of the background to exclude false-positive results; as MRD low positive if positivity was less than 5×10^{-4} or below the achieved quantitative range (ie, not quantifiable); or as MRD high positive if the positivity was $\geq 5 \times 10^{-4}$, the threshold used in the AIEOP-BFM ALL 2000 study to define HR-MRD levels after induction and consolidation.

RESULTS

Study Population

The main features of the 110 study patients are summarized in Table 1. The analysis found a strong association between higher PCR MRD levels at the two AIEOP-BFM ALL 2000 protocol TPs (TP1, day +33; TP2, day +78) and the frequency of MRD positivity at later TPs; patients with favorable genetic features (hyperdiploidy or TEL-AML positivity) and B-cell precursor immunophenotype were less likely (although not significantly) to have MRD positivity at later TPs. Table 2 depicts the outcome of treatment: at a median follow-up time of 9.8

Table 1. Main Characteristics of 110 Patients With Childhood ALL Who Underwent PCR-MRD Monitoring Performed During and After Treatment Completion

Characteristic	Total		PCR-MRD: Negative		PCR-MRD: Low Positive ($< 5 \times 10^{-4}$)		PCR-MRD: High Positive ($\geq 5 \times 10^{-4}$)		*P*
	No.	%	No.	%	No.	%	No.	%	
Total No. of patients	110		81	73.6	23	20.9	6	5.5	
Sex									.74
Male	54	49.1	39	48.2	12	52.2	3	50.0	
Female	56	50.9	42	51.8	11	47.8	3	50.0	
Age (years)									1.0
1-9	93	84.6	68	84.0	20	87.0	5	83.3	
10-17	17	15.4	13	16.0	3	13.0	1	16.7	
WBC count (per microliter)									.38
$<$ 100,000	103	93.6	77	95.1	21	91.3	5	83.3	
$\geq$ 100,000	7	6.4	4	4.9	2	8.7	1	16.7	
Phenotype									.24
T-ALL	9	8.2	5	6.2	2	8.7	2	33.3	
BCP-ALL	101	91.8	76	93.8	21	91.3	4	66.7	
Hyperdiploidy or TEL/AML1 positive									.10
Yes	50	48.5	41	53.2	7	35.0	2	33.3	
No	53	51.5	36	46.8	13	65.0	4	66.7	
Not known	7		4		3		0		
Response to PDN									1.0
PGR	106	96.4	78	93.8	23	100.0	5	83.3	
PPR	4	3.6	3	3.7	0		1	16.7	
PCR-MRD level on day +33									.009
Negative	48	44.0	40	49.4	8	34.8	0		
Low positive	42	38.5	32	39.5	9	39.1	1	20.0	
High positive	19	17.5	9	11.1	6	26.1	4	80.0	
Not known	1		0		0		1		
PCR-MRD level on day +78									$<$ .001
Negative	87	79.1	73	90.1	14	60.9	0		
Low positive	20	18.2	8	9.9	8	34.8	4	66.7	
High positive	3	2.7	0		1	4.3	2	33.3	
Final stratification by AIEOP-BFM ALL 2000 protocol									.15
Standard risk	42	38.2	35	43.2	7	30.4	0		
Intermediate risk	61	55.4	42	51.8	15	65.2	4	66.7	
High risk	7	6.4	4	4.9	1	4.4	2	33.3	

Abbreviations: AIEOP-BFM ALL 2000, International Collaborative Treatment Protocol for Children and Adolescents With Acute Lymphoblastic Leukemia; ALL, acute lymphoblastic leukemia; BCP-ALL, B-cell precursor ALL; MRD, minimal residual disease; PCR, polymerase chain reaction; PDN, prednisone; PGR, prednisone good responder; PPR, prednisone poor responder; T-ALL, T-cell ALL.

Table 2. Treatment Outcome of 110 Patients With Childhood ALL, According to the Results of PCR-MRD Monitoring Performed After Induction-Consolidation Treatment

Outcome	Total		PCR-MRD: Negative		PCR-MRD: Low Positive ($< 5 \times 10^{-4}$)		PCR-MRD: High Positive ($\geq 5 \times 10^{-4}$)	
	No.	%	No.	%	No.	%	No.	%
Total No. of patients	110		81		23		6	
First remission	87	79.1	72	88.9	14	60.9	1	16.7
Relapsed	23	20.9	9	11.1	9	39.1	5	83.3
Site								
BM isolated	17		6		6		5	
BM combined	3		1		2		0	
Extramedullary	3		2		1		0	

Abbreviations: ALL, acute lymphoblastic leukemia; BM, bone marrow; MRD, minimal residual disease; PCR, polymerase chain reaction.

years, 23 patients (20.9%) had developed a relapse isolated in the BM (n = 17), in an extramedullary site (n = 3), or in a combination of the two (n = 3), either during or after completion of treatment. All relapses showed the same gene rearrangement already identified at diagnosis. The remaining 87 patients (79.1%) were in first CR at the time of last follow-up. The probability of 5-year DFS was 81.8% (SE, 3.7%), with a CIR of 18.2% (SE, 3.7%). Their probability of 5-year DFS was not different from that of the whole AIEOP-BFM ALL 2000[6,10] cohort of patients in first CR after induction IA (Appendix Fig A1, online only).

Association Between MRD Results and Outcome

A total of 588 samples were analyzed, with a median of five samples per patient. The molecular markers analyzed are listed in Appendix Table A1 (online only). Overall, of the 588 samples, 539 (91.7%) were classified as negative, 41 (7%) as MRD low positive, and eight (1.4%) as MRD high positive based on at least one marker.

Of the 110 patients included in this study, six (5.5%) had one or more MRD high-positive results during the observation time, and five of them subsequently relapsed at 1, 3, 3, 8, and 12 months from the first high-positive result (Table 2 and Appendix Fig A2 [online only]). Thus, the positive predictive value (ie, the probability of relapsing given an MRD high-positive finding after day +78) was 83%. A total of 23 patients were classified as low positive at one or more TPs; of those patients, nine relapsed at a median of 18 months after the initial positive finding, with a positive predictive value of 39%; the median follow-up after first positivity of the remaining 14 patients in CR was 9.8 years. A total of 81 patients were MRD negative at all tested TPs; of them, nine relapsed, indicating that the probability of remaining in remission, given that MRD never reached positivity during postinduction treatment (ie, the negative predictive value) was 89%.

The rate of relapse was similar for patients with T-lineage (two of nine patients) or B-lineage (21 of 101) ALL. Among patients with B-lineage ALL, all four patients with high MRD positivity relapsed, accounting for 20% of all relapses. Of interest, of three patients with B-lineage ALL who developed an isolated extramedullary relapse soon after stopping therapy, one had a single low-positive result at an initial TP and tested negative thereafter, and the other two always tested MRD negative (Appendix Fig A2).

The 5-year CIR was 8.6% (SE, 3.1%) for the MRD-negative patients, 34.8% (SE, 9.9%) for the patients who had at least one low-positive result, and 83.3% (SE, 15.2%) for the patients who had at least one high-positive result (Fig 1). Thus, detection of MRD positivity was significantly related to the risk of relapse ($P < .001$).

We also evaluated, within the larger subgroup of 96 patients with B-lineage ALL without HR features, whether the MRD profile was predictive of the risk of relapse after adjusting for risk group (SR *v* IR) in a Cox model. Patients with at least one low-positive (hazard ratio, 4.3; 95% CI, 1.53 to 12.0; $P = .006$) or high-positive (hazard ratio, 21.2; 95% CI, 4.9 to 91.0; $P < .001$) value had a significantly higher risk of relapse.

To assess the added value of short-term versus long-term MRD monitoring, we separately analyzed patients who had MRD positivity (n = 18) or negativity (n = 86) shortly after the induction-consolidation phases (ie, within 4 months after day +78; six patients were not evaluated for early MRD). Of the 18 patients with early MRD positivity, one relapsed early (HR patient 108); 12 tested repeatedly positive at serial MRD measurements, and five of them relapsed; five subsequently tested MRD negative, but three of them eventually relapsed, too; thus the total number of relapses in this subgroup was nine (50%). Interestingly, one patient with T-cell ALL (T-ALL) repeatedly tested positive up to the end of treatment; nevertheless, he remained in CR for 12 years after the diagnosis (IR patient 99).

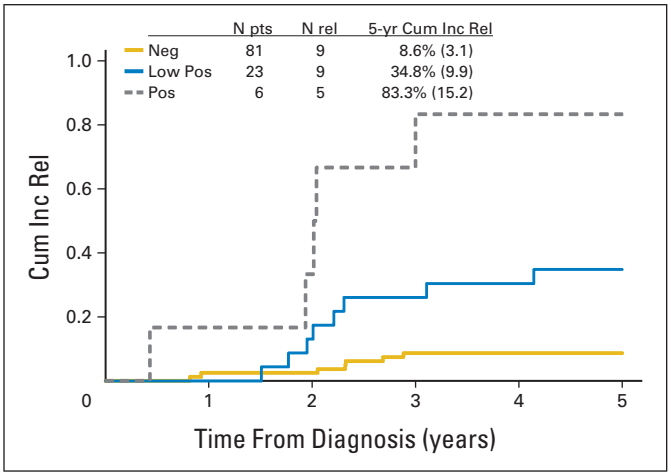

Fig 1. Cumulative incidence of relapse (Cum Inc Rel) in 110 children with acute lymphoblastic leukemia, according to the results of polymerase chain reaction monitoring of minimal residual disease during postinduction treatment. Numbers in parentheses represent SE. Neg, negative; Pos, positive.

Of the 86 patients who were found to be MRD negative in the 4 months following the induction-consolidation phases, 77 (90%) remained negative thereafter at all the TPs tested, and nine (10%) eventually relapsed; the remaining nine patients instead showed MRD positivity at one (or more) following TPs, and four of them relapsed. Of the six patients who lacked early MRD follow-up, two showed subsequent MRD positivity, and one of them relapsed.

DISCUSSION

The prognostic value of MRD in childhood ALL has been firmly established by several groups worldwide. In particular, the use of RQ-PCR MRD has been widely documented by the AIEOP-BFM clinical trials as feasible and reproducible.[6,10] The issue of MRD monitoring at later TPs has been considered since the early 1990s. In the pioneering study by Van Dongen et al,[5] any MRD positivity at any single TP during first-line treatment was predictive of poor outcome. However, because of the high predictive value of combined MRD results at the first two TPs and because of the rarity of positivity at later TPs, systematic MRD monitoring after induction-consolidation therapy was considered to be not cost-effective. In 2003, the Australian and New Zealand Children's Cancer Study Group reported the results of MRD monitoring at 1 and 2 years from diagnosis, in which patients with positive MRD underwent treatment intensification.[25] The minority of patients with late MRD positivity had a poor outcome despite

MRD-directed treatment intensification. Their data allowed the conclusion that systematic MRD monitoring at late TPs could not be recommended.[26] In our study, we explored the application of extended, prospective PCR MRD monitoring beyond the two TPs used for patient stratification in the AIEOP-BFM studies. The results are strongly predictive of the individual patient's outcome: indeed, patients with high-positive levels ($\geq 5 \times 10^{-4}$) had a high risk of developing a BM relapse during treatment (five relapses in six patients). This compares with a 39% relapse rate in patients with a single or repeated MRD low-positive result ($< 5 \times 10^{-4}$) and an 11% relapse rate in patients with a negative MRD profile.

These findings are in keeping with the results obtained by Raff et al[20] in the GMALL (German Multicenter Trial for Treatment of Newly Diagnosed Acute Lymphoblastic Leukemia in Adults) 06/99 trial and the GMALL 07/2003 trial in which conversion to MRD positivity during the early postconsolidation phase in adult patients with SR-ALL was highly predictive of subsequent hematologic relapse. Yet our data are only partially in keeping with the recent experience of the German Multicenter Study Group for Adult ALL, documenting that patients in molecular CR after consolidation had a significantly higher probability of both overall survival and DFS compared with patients with evidence of molecular relapse. However, in the adult setting, patients with molecular relapse without transplantation in first CR had a median time to cytologic relapse of 2.6 months from MRD positivity and a probability of continuous complete remission of only 5%.[27] In our experience, the probability of continuous complete remission was 60% in patients (n = 9) who tested positive only at later TPs and 50% in patients (n = 18) who had a molecular positivity early (within 4 months) after induction-consolidation therapy. Although definitely better than the outcome in adult patients, the outcome of this last group is considered inadequate compared with that of the general population of patients with childhood ALL. They account for 16% (18 of 104) of the patients and can be identified by a relatively low-cost MRD monitoring at a single additional TP within 4 months after induction and consolidation therapy. Monitoring appears to be indicated, especially in patients who test MRD positive at day +78 via PCR: among 21 such patients, 11 remained MRD positive, and five of them relapsed. Early therapeutic interventions with alternative agents and/or with allogeneic HSCT in first CR appears to be indicated. Otherwise, systematic MRD monitoring in 86 patients who were MRD negative within 4 months after induction-consolidation therapy allowed the identification of only nine patients with MRD positivity later on, and four of them relapsed (three close to stopping therapy, one at 7.5 years after diagnosis). It should also be noted that nine relapses occurred in 77 patients (12%) in whom no positive signals were detected.

The finding of persistence of MRD-positive results throughout continuation chemotherapy, despite continuous CR in one patient, is puzzling. This patient had IR T-ALL, with adequate initial response (good response to steroid prephase) and low MRD positivity at TP2. Because (by study design) the attending physicians and the family were blinded to the results of the MRD analysis at later TPs, this patient did not receive any additional treatment intensification or extension. MRD measurements after TP2 had never been done in the cooperative AIEOP-BFM group outside this study. In patients with T-ALL and comparable MRD levels in the AIEOP-BFM ALL 2000 study, the probability of remaining relapse-free was 74%.[10] The pattern of MRD in this patient might represent the tip of the iceberg if MRD levels fluctuate around and below the threshold of detection; if so, cure for ALL might not necessarily result from complete eradication of the disease. This hypothesis might also explain why patients may occasionally test low positive throughout maintenance therapy without developing relapse, as observed in our study, and it confirms that the definition of molecular relapse remains quite intriguing.[18,19] In this context, the risk of false MRD positivity should also be considered. This risk is higher for low levels of MRD, and for this reason we applied the EuroMRD guidelines in a more conservative way, as explained in Patients and Methods. Theoretically, false MRD positivity could also occur in the case of persistence of the preleukemic genetic aberration[28] or of the chromosomal lesion in a nonappropriated target cell.[29] Our patient 99 was actually monitored by using the SIL-TAL genomic break point marker, in which a putative preleukemic origin of the translocation cannot be ruled out.

Yet the unsolved dilemma remains on whether available technologies are able to define premorphologic relapse at a time interval which may become suitable for useful clinical intervention. It is interesting to remember that, in the past, an attempt to herald leukemia by the peripheral cell blood count did not result in any therapeutic advantage for the children and was thus abandoned.[30] In addition, it should be considered that systematic MRD monitoring implies a major investment of resources and an important psychological burden on patients and families. These aspects deserve attention when drawing conclusions about the results of protracted MRD monitoring.

In our opinion, our data suggest that additional MRD evaluation within a few months after induction and consolidation therapy may be clinically justified, particularly in patients who did not reach MRD negativity, because it allows identification of a nonnegligible fraction of patients who have a high risk of relapse. This is important in non-HR patients, in whom it is worth investigating whether molecular slow responders may have an advantage from an early intervention. In HR patients, this strategy may provide useful information to define the timing of HSCT or to assess the benefit of specific therapeutic interventions. Systematic monitoring of MRD in patients who reach MRD negativity by the end of induction-consolidation therapy appears to be not justified in our experience.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

The author(s) indicated no potential conflicts of interest.

AUTHOR CONTRIBUTIONS

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Manuscript writing: All authors
Final approval of manuscript: All authors

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Appendix

Treatment Protocol

All patients were given 7 days of prophase therapy, including steroid therapy (prednisone) and one intrathecal dose of methotrexate (MTX), followed by induction protocol IA and induction consolidation protocol IB; on day 8, patients were randomly assigned to continue steroid treatment with either prednisone (60 mg/m^2 per day) or dexamethasone (10 mg/m^2 per day) until day 28 (the dose of steroid [prednisone or dexamethasone] was tapered at the end of the time interval for full-dose assumption). Standard-risk patients received four courses of high-dose MTX (2 g/m^2; protocol M) and intermediate-risk patients received 5 g/m^2; protocol M), oral 6-mercaptopurine, and intrathecal therapy. At the beginning of the reinduction phase, a second randomization was planned: for standard-risk patients, protocol II versus reduced-intensity protocol III; for intermediate-risk patients, protocol II versus reduced-intensity protocol III given twice; for high-risk patients, three blocks of non–cross-resistant drugs followed by protocol III given three times versus protocol II given twice. Maintenance therapy consisted of 6-mercaptopurine once per day and MTX once per week until 24 months from diagnosis. CNS-directed therapy consisted of intrathecal MTX during each treatment phase including continuation unless cranial radiotherapy (dosage by age) was given to the following patients: high-risk patients age 2 years or older, or those with non–high-risk T-ALL and leukocyte count of more than 100,000/μL at diagnosis, or those with CNS involvement.

Table A1. Molecular Markers

Patient	Marker	Quantitative Range	Sensitive Range	Present in the Relapse
1	VH3JH4	1×10^{-4}	1×10^{-5}	X
2	VH6JH5	5×10^{-4}	1×10^{-5}	X
3	VKIIKDE	5×10^{-4}	1×10^{-5}	X
4	VH1JH4	1×10^{-3}	1×10^{-4}	X
5	DH5JH5	1×10^{-3}	1×10^{-5}	X
6	VH3JH6	5×10^{-4}	1×10^{-5}	X
7	VH1JH6	1×10^{-4}	1×10^{-5}	Yes
8	VH1JH6	1×10^{-4}	1×10^{-5}	Yes*
9	VH2JH6	1×10^{-3}	1×10^{-5}	X
10	VKIIKDE	1×10^{-3}	1×10^{-5}	Yes
11	VH1JH4	5×10^{-4}	1×10^{-4}	X
12	VH3JH5	1×10^{-3}	1×10^{-4}	X
13	DH4JH6	5×10^{-4}	1×10^{-5}	X
14	VD2DD3	1×10^{-4}	1×10^{-4}	X
15	VH4JH4	1×10^{-4}	1×10^{-5}	X
16	VH3JH1	5×10^{-4}	1×10^{-4}	X
17	VH6JH4	5×10^{-4}	1×10^{-5}	X
18°	VH3JH6	ND†	1×10^{-4}	Yes
19	VH4JH2	1×10^{-4}	1×10^{-5}	X
20	VKIKDE	1×10^{-4}	1×10^{-5}	X
21	VD2JD1	1×10^{-3}	1×10^{-4}	X
22	VH3JH6	5×10^{-4}	1×10^{-4}	X
23	VH3JH6	1×10^{-4}	1×10^{-5}	X
24	VH3JH4	1×10^{-4}	1×10^{-5}	X
25	VH3JH6	5×10^{-4}	1×10^{-4}	X
26	VH3JH5	1×10^{-4}	1×10^{-5}	X
27	DH6JH4	5×10^{-4}	1×10^{-4}	X
28	VH1JH5	1×10^{-4}	1×10^{-5}	X
29	VH3JH4	1×10^{-3}	1×10^{-5}	X
30	VD2DD3	1×10^{-4}	1×10^{-4}	X
31	VH4JH6	1×10^{-5}	1×10^{-5}	X
32	VH4JH5	1×10^{-4}	1×10^{-5}	X
33	DD2DD3	5×10^{-4}	1×10^{-4}	X
34	VH3JH6	5×10^{-4}	1×10^{-5}	X
35	VH3JH4	1×10^{-4}	1×10^{-5}	X
36	VH3JH4	1×10^{-4}	1×10^{-4}	X
37	VH6JH4	1×10^{-4}	1×10^{-5}	X
38	VH7JH6	1×10^{-3}	1×10^{-4}	X
39	VH3JH6	1×10^{-3}	1×10^{-5}	X
40	VH6JH6	5×10^{-4}	1×10^{-4}	X

(continued on following page)

Table A1. Molecular Markers (continued)

Patient	Marker	Quantitative Range	Sensitive Range	Present in the Relapse
41	VH1JH4	1×10^{-4}	1×10^{-5}	X
42	VH1JH4	1×10^{-3}	1×10^{-5}	X
43	VKIIIKDE	1×10^{-4}	1×10^{-5}	Yes*
44	VH3JH6	5×10^{-4}	1×10^{-5}	X
45	VH3JH4	1×10^{-4}	1×10^{-5}	X
46	VH2JH4	1×10^{-3}	1×10^{-5}	X
47	VH4JH4	1×10^{-4}	1×10^{-5}	Yes
48	VH1JH6	1×10^{-4}	1×10^{-5}	X
49	VH4JH6	1×10^{-4}	1×10^{-4}	X
50	VD1JD1	1×10^{-3}	1×10^{-5}	X
51	VH4JH6	1×10^{-4}	1×10^{-5}	X
52	VH3JH3	5×10^{-4}	1×10^{-5}	X
53	VH3JH3	1×10^{-4}	1×10^{-5}	X
54	VD2DD3	1×10^{-4}	1×10^{-5}	X
55	VH3JH6	1×10^{-4}	1×10^{-5}	X
56	VKIKDE	5×10^{-4}	1×10^{-5}	X
57	VD2DD3	1×10^{-3}	1×10^{-5}	X
58	VD2DD3	1×10^{-3}	1×10^{-4}	X
59	VH3JH4	1×10^{-4}	1×10^{-4}	X
60	VD2DD3	5×10^{-4}	1×10^{-5}	X
61	VB6.4JB1.5	5×10^{-4}	1×10^{-4}	X
62	VH3JH5	1×10^{-3}	1×10^{-4}	X
63	VH4JH4	1×10^{-3}	1×10^{-5}	Yes
64	VKIIKDE	1×10^{-3}	1×10^{-4}	X
65	VH4JH4	1×10^{-4}	1×10^{-5}	X
66	VH3JH4	5×10^{-4}	1×10^{-5}	X
67	VH3JH4	1×10^{-4}	1×10^{-5}	X
68	VD2DD3	1×10^{-3}	1×10^{-4}	Yes
69	VH3JH5	5×10^{-4}	1×10^{-4}	X
70	VH3JH5	1×10^{-3}	1×10^{-4}	X
71	VH2JH6	5×10^{-4}	1×10^{-5}	X
72	VH3JH4	1×10^{-4}	1×10^{-5}	Yes
73	VKIKDE	1×10^{-3}	1×10^{-4}	X
74	VH1JH6	1×10^{-4}	1×10^{-5}	X
75	VH3JH4	1×10^{-3}	1×10^{-4}	X
76	VH5JH6	5×10^{-4}	1×10^{-5}	X
77	VH1JH6	5×10^{-4}	1×10^{-4}	X
78	DH6JH6	1×10^{-5}	1×10^{-5}	X
79	VD2DD3	1×10^{-3}	1×10^{-5}	Yes
80	DH3JH6	5×10^{-4}	1×10^{-4}	Yes
81	DH6JH4	1×10^{-4}	1×10^{-5}	X
82	VD1JD1	5×10^{-4}	1×10^{-5}	X
83	DH4JH6	5×10^{-4}	1×10^{-5}	X
84	VH3JH6	1×10^{-4}	1×10^{-5}	X
85	VH3JH5	1×10^{-4}	1×10^{-4}	X
86	VH1JH6	1×10^{-4}	1×10^{-5}	X
87	DD2DD3	1×10^{-3}	1×10^{-4}	X
88	VH2JH6	1×10^{-3}	1×10^{-4}	Yes*
89	VH1JH4	5×10^{-4}	1×10^{-4}	Yes
90	VKIIKDE	1×10^{-3}	1×10^{-4}	X
91	VH6JH5	5×10^{-4}	1×10^{-4}	Yes
92	VH3JH6	5×10^{-4}	1×10^{-4}	Yes
93	VD1JD1	1×10^{-3}	1×10^{-5}	X
94	VD2DD3	5×10^{-4}	5×10^{-4}	X
95	DH6JH4	1×10^{-3}	1×10^{-4}	X
96	VH1JH2	5×10^{-4}	1×10^{-5}	X
97	VD2DD3	5×10^{-4}	1×10^{-5}	X
98	VD2DD3	5×10^{-4}	1×10^{-4}	X
99	SILTAL	5×10^{-4}	1×10^{-5}	X

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Table A1. Molecular Markers (continued)

Patient	Marker	Quantitative Range	Sensitive Range	Present in the Relapse
100	VH3JH6	1×10^{-4}	1×10^{-5}	Yes
101	VH1JH4	1×10^{-3}	1×10^{-4}	Yes
102	VD2DD3	1×10^{-4}	1×10^{-5}	X
103	VH3JH4	1×10^{-3}	1×10^{-4}	Yes
104	VH2JH4	5×10^{-4}	5×10^{-4}	Yes‡
105	VH3JH3	5×10^{-4}	1×10^{-5}	X
106	VD2DD3	1×10^{-4}	1×10^{-5}	X
107	VG8JG1.3	1×10^{-4}	1×10^{-5}	Yes
108	VH6JH4	1×10^{-4}	1×10^{-4}	Yes
109	VD1JD1	1×10^{-4}	1×10^{-4}	Yes
110	DH7	1×10^{-3}	1×10^{-5}	Yes

Abbreviations: ND, not determined; X, not tested, the patient did not relapse.
*Low positivity in the bone marrow.
†It was not possible to determine the QR for the patient on 7700. The marker was acquired on 7700 Sequence Detection System (Applied Biosystems); we could not re-analyze the data according to the guidelines[24] to give the quantitative range so we define only the sensitive range.
‡Patient 104 partially modified the rearrangement; the MRD primer was designed in the conserved N-region between the DH and JH.

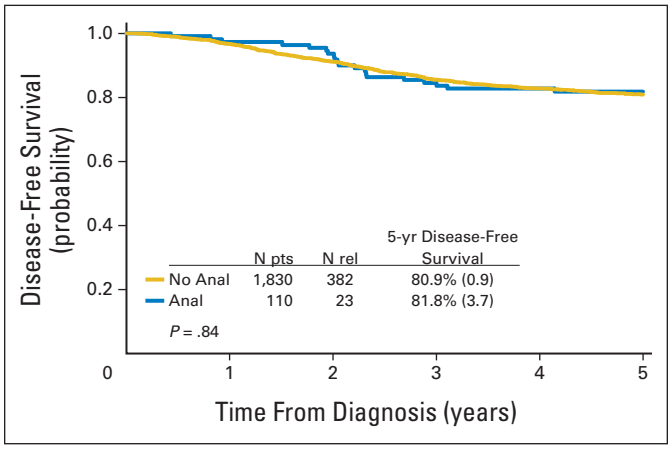

Fig A1. Disease-free survival of the 110 patients selected for this study compared with that of the total study population of the International Collaborative Treatment Protocol for Children and Adolescents With Acute Lymphoblastic Leukemia (AIEOP-BFM ALL 2000) who reached complete remission by the end of phase IA. Our total study population had an outcome that was comparable to that of the overall AIEOP-BFM ALL 2000 study population. Numbers in parentheses represent SE. No Anal, no analysis; pts, patients; rel, relapse.

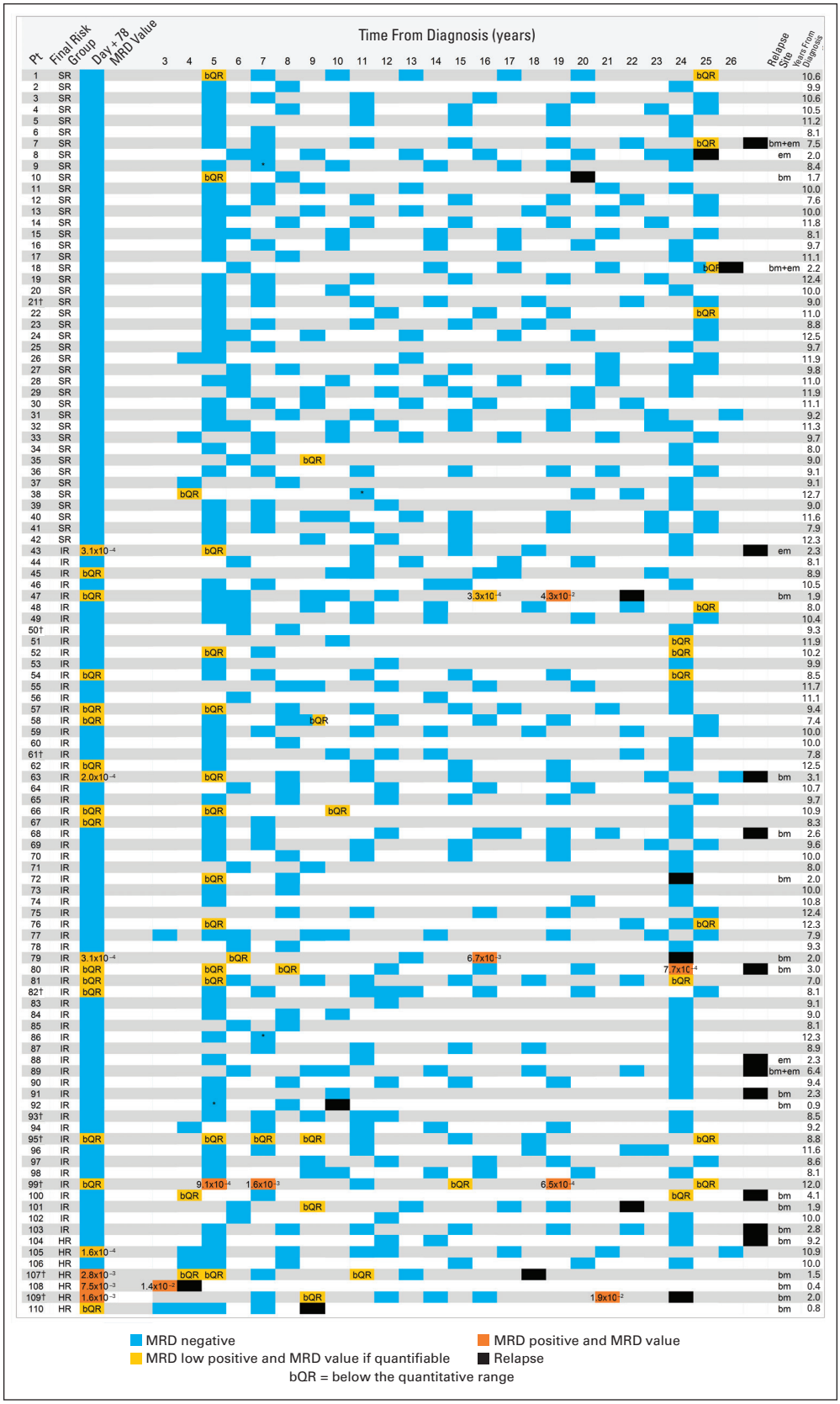

Fig A2. Results of the postinduction monitoring of bone marrow minimal residual disease (MRD) in individual study patients. bm, bone marrow; em, extramedullary; HR, high risk; IR, intermediate risk; SR, standard risk. (*) 2 bm samples. (†) T lineage; all other cases were of B lineage.